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We are approximately one year into the funding of this proposal. To date, we have acquired images from 30 patients. They include (a) gray scale images, (b) color Doppler images and (c) power Doppler images. Tissue samples were collected from 27 patients. Three patients did not opt to go for surgery. The images from the patients acquired so far have been completely digitized and analyzed to determine the extent of vascularity. The software necessary to make these measurements is now fully functional and in place for making the measurements on a routine basis. Tissue staining and microvessel count have been completed on thirteen pateints. A preliminary analysis comparing vascularity determined by Doppler imaging with that measured by histologic analysis is encouraging. These results are discussed below and will be presented at the New Era of Hope meeting, scheduled for October 1997. There is an excellent agreement between the real color levels of Doppler images and histologic vessel count. In particular, both histologic and image analysis show (1) the malignant cancers are more vascular than the benign lesions; and (2) the periphery of the lesion is more vascular in the malignant cancer as well as the benign lesions. These preliminary results suggest that Doppler imaging can potentially be used for noninvasive differentiation of the malignant from the benign. Furthermore, the quantitative scheme of measuring vascularity that will come from this research could potentially be used to noninvasively monitor the response of tumor to treatments of patients in clinical settings. A complete analysis of the data at the end of the second year of this proposal will provide a better assessment of these possibilities.

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Table of Contents

Introduction

Many studies have shown a link between tumor growth patterns and the distribution of microvessels in the tissues. The growth of a solid tumor beyond a size of 2 mm (about 10^6 cells) requires a continual neovascularization to supply oxygen and nutrients to the cells [1,2]. The increase in the number of new capillaries also implies a higher probability for the tumor cells to enter blood circulation, thus creating a greater likelihood of metastasis [3]. There is convincing evidence that microvessel density (# of vessels per unit area of the tissue) is predictive of the metastatic disease in breast carcinoma [2,4-6], and an independent prognostic indicator of cancer reoccurrence in patients with node-negative carcinoma [7]. Thus, in the evaluation of breast cancer the role of the growth and the development of microvessels (angiogenesis) must also be considered. The difficulty in including these factors in the routine clinical examination is that the current methods of assessing angiogenesis can only be used on surgical samples. A technique that can non-invasively image microvessels and provide an estimate of its density is highly desirable. A successful development of such a technique could have a beneficial impact on the evaluation and the management of patients with breast cancer. This would not only help in selecting patients with high risk, but may assist in differential diagnosis, and also aid in monitoring the changing status of the disease as a result of therapeutic interventions.

We believe ultrasound imaging, which allows direct visualization of blood vessels, is a strong candidate to achieve this goal. The purpose of this study is to determine the potential of Doppler ultrasound imaging in evaluating vascularity of the breast in patients with suspicious masses. Specifically the goals of this study are as follows:

1. Acquire Color and power Doppler images from a total of 75 patients who are scheduled for surgery for breast cancer.
2. Use these images and computer analyses to determine the extent of vascularity.
3. Make histologic measurements of microvessel density in the breast masses removed during surgery.
4. Use linear and non-linear univariate/multivariate regression analyses to establish quantitative relationships between:
   (a) color and power Doppler measurements
   (b) microvessel density and color Doppler measurements
   (c) microvessel density and power Doppler measurements
5. Test the ability of the various quantitative relationships to predict microvessel density from color and power Doppler measurements.

Methods and Results

The proposed work was performed by a team of researchers at the University of Pennsylvania. Dr. Sehgal PhD coordinated the overall study, organized the experimental protocols, and carried out computer image analyses; Dr. Sullivan MD, selected the patients suitable for the proposed study; Drs. Arger MD and Rowling MD performed color and power Doppler imaging by using state of the art imaging equipment; and Dr. Reynolds performed histologic classification of tissues and microvessel density analysis. Dr. Emily Conant has joined the Department of Radiology, University of Pennsylvania, as the Chief of Breast Imaging section. She will be taking over the duties of Dr. Sullivan on this project for the year 1997-1998.

PATIENT SELECTION

This study was performed on patients who chose to undergo breast surgery at the Hospital of the University of Pennsylvania. Patients with a suspicious breast mass, either

benign or malignant (primary invasive breast tumor, Stage T1 to T3) with no other previous primary cancer were selected for the study. Ultrasound imaging was performed immediately after mammography and before any diagnostic procedure (fine needle aspiration, core biopsy, or excisional bx) to avoid any disturbance of blood flow. For each patient the following clinico-pathologic characteristics were recorded: age, histologic type, tumor size and grade.

DOPPLER IMAGING

Ultrasonic and Doppler (color flow and power) imaging was performed by using ATL3000 (ATL, Bothell, WA) scanner currently installed and in use in our facilities. The breast was first scanned in B-mode. The plane representing the largest cross-section of the lesion was identified and the diameter, D_1, measured. The diameters in the two orthogonal planes, D_2 and D_3 will also be measured and the volume V determined by the ellipsoid formula: $V=0.5*D_1*D_2*D_3$. This measurement is used as one of the clinico-pathological characteristics. The color Doppler images were recorded on a video tape without aliasing at the lowest possible wall filter. The Doppler gain was increased until background "noise" appears across the image and backed off until few random specks are visible [9]. The term noise means appearance of color in the image without apparent flow. The patients were asked to hold the breath for 2-3 seconds during the image recording. Images were recorded in at least three different planes (five different planes when the size of the lesion was large) for each patient. Following this, power Doppler images were recorded in 3 to 5 different planes. All the image parameters, except color gain, were kept constant in all our studies.

COMPUTER IMAGE ANALYSES

The color and power Doppler images were digitized from the video tapes at 24 bit resolution. The images were analyzed by software developed by us at the University of Pennsylvania. The user outlines the lesion on an image. The computer determines the area of the lesion and draws a region in the center of the lesion with half the total area. On each of these regions, referred from here on as central and peripheral regions, color analysis is made by the following procedure: the software reads a palette bar displayed on the images and the user assigns a value of 0 to the "lowest" and a value of 100 to the "highest" color in the palette bar. With this information the computer program constructs a "look up" table from the hue, saturation, and brightness values of the colors present in the palette bar. Using the look-up table, the computer identifies the colored pixels within the region outlined by the user as a tumor. It counts the number of colored pixels (n) and the number of pixels identified as tumor (N). It also measures the color level of each of the pixels, which represents the characteristics of flow. If i represents a color pixel, and C_i its color level, then the vascular density (VD) and mean color level (MCL) can be defined by the formulas

$$VD = \frac{100 * \sum_{i}^{n} i}{N} = \%\ \text{area of the lesion occupied by blood vessels, and}$$

$$MCL = \frac{\sum_{i}^{n} C_i i}{Gn},$$

respectively, where G represents the scaling factor, with values ranging from 0 to 1, for the color gain used during imaging. The magnitude of MCL represents different characteristics of the color and power Doppler images. In color Doppler images it represents mean local

flow velocity, and on power Doppler images it represents the number of red blood cells moving above a threshold velocity. If one assumes local hematocrit in these small blood vessels to be equal to systemic hematocrit, MCL can be regarded to be related to blood volume (or, more appropriately related to the log of blood volume because the signals are often log compressed) moving above a threshold velocity. The relationship between MCL and physiological parameters (mean flow velocity and blood volume) should be viewed to be semi-quantitative because of several variables: the angle between a blood vessel and the direction of ultrasound; the choice of scale maximum, filters and color write priority threshold; and, the interpolation, averaging and other image processing algorithms used internally within a scanner to display images for optimal viewing. However, if the imaging parameters are kept constant throughout the study, the influence of these variables can be significantly reduced and under these circumstances the data provides a meaningful comparison of the flow characteristics through the benign and the malignant lesions. Several studies in the last two years have demonstrated that this can indeed be achieved by careful control of image parameters [13-15].

TUMOR COLLECTION AND HISTOLOGY

The surgical breast specimens were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 mm thickness in accordance with standard methods and stained with hematoxylin-eosin (H&E). These specimens were examined and representative sections (maximum of three) of the tumor were selected for quantitation of microvessel density (see microvessel staining, grading and counting). The WHO classification by Azzopardi [11] was used to identify the histologic type of the breast tumor. The histologic grading was performed on H&E sections according to the Elston modification of the Bloom and Richardson criteria [12]. This grading system combines three morphologic features of infiltrating breast carcinomas into a final grade [extent of glandular differentiation, nuclear grade and highest mitotic count in a representative area of 10 high-powered fields (40X objective, 10X ocular)].

MICROVESSEL STAINING, GRADING, AND COUNTING

As mentioned in Tumor Collection and Histology, the specimens were examined with H&E stained sections to select three representative areas of the primary tumor for quantitation of microvessel density. Each area selected was sectioned at 5 mm and the microvessels were highlighted by staining endothelial cells for factor VIII-related antigen and CD31 using a standard immunoperoxidase staining technique. Microvessel density was determined in the areas of tumor containing the highest numbers of capillaries and small venules (microvessels) per most intense neovascularization [2]. Briefly, these neovascularized "hot spots" were detected by scanning the primary tumor sections at low power (4X and 10X) and identifying the area of most intense neovascularization. Individual microvessel counts were then be made on a 200X field (20X objective, 10X ocular) within the tumor "hot spot". Microvessels considered were individual endothelial cells or endothelial cell clusters that stain positive for factor VIII-related antigen and/or CD31. Results were expressed as the highest number of microvessels identified within any single 200X field and the average count of microvessels in the three sections were recorded for each antibody. The microvessel density and average number of microvessels were correlated with the Doppler imaging results. The results from Doppler imaging were not known to the pathologist assessing microvessel density.

SAMPLING ERRORS

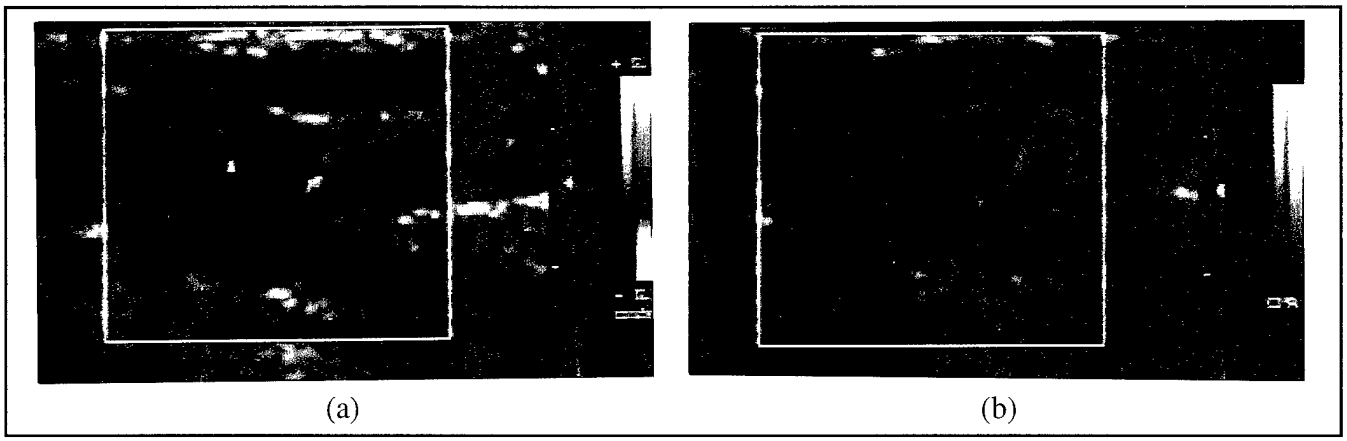
Under ideal conditions the imaging and histology measurements must be made at exactly the same site of the tumor. In a real situation it is not feasible to match the two

sampling sites. This can lead to differences in measurements because the arterial and capillary networks are complex, asymmetric, and unevenly spaced over the cancer volume [10]. To minimize the sampling variations the MVD and imaging measurements were made in several planes and the mean of these values were be used for establishing correlative relationship.

Results

To date, we have imaged 30 patients. The mean age of the patients is 57 $\pm$ 15 years and ranges between 35 -79 years. Of these 30 patients 17 are Caucasians, 9 are Afro-American and 4 are unknown. Tissue samples were collected from 27 patients. Three patients did not to go for surgery. The images from all the patients have been completely digitized and analyzed to determine the extent of vascularity. Tissue staining and microvessel count has been completed on 13 patients and correlated with the imaging data. The histology on the remaining 14 patients is also in and we are in the process of analyzing this data.

Seven masses were characterized as malignant including 3 infiltrating ductal carcinomas, 2 in situ and infiltrating ductal carcinomas, 1 lobular carcinoma in situ and 1 mixed ductal/lobular carcinoma. The size of the lesions ranged from 0.1 - 9 ml (average $\pm$ std, 1.10 $\pm$ 1.96 ml). Blood vessels were detected with color Doppler (CoD) imaging in all the cases and 12/13 (92%) cases with power Doppler (PoD). Figure 1a shows a CoD image of a patient with infiltrating ductal carcinoma which appears as a hypoechoic region (0.8 cm x 0.6 cm x 0.8 cm) in the B-scan image. The blood vessels, shown in color, can be seen traversing through the center as well as through the periphery of the lesion. The scale of mean velocity, shown on the right as a palette bar, is $\pm$ 6.4 cm/sec. The color blue and red also seen in the blood vessel represent slow flow and is on the order of 1 cm/sec. The width of the blood vessels is on the order of 1 mm. This image has a close correspondence with Figure 1b showing the PoD image of same lesion. The PoD image also shows blood vessels traversing through the core of the lesion as well as in the peripheral region. The weak scattered coloration seen in the region of interest is due to the "flash artifact" caused by tissue and/or transducer motion.



(a) (b)

Figure 1: Doppler images of a patient with infiltrating carcinoma: (a) color Doppler image, (b) power Doppler image.

Figure 2 shows a very different flow pattern. It shows CoD and PoD images of a patient with benign phyllodes tumor (2 cm x1.9 cm x 2 cm). In either case no blood vessels are detected within the mass of the tumor. Small blood vessels can be seen at the periphery of the lesion. Other benign cases showed greater flow within the lesion than seen in b, but in each case it was markedly subdued.

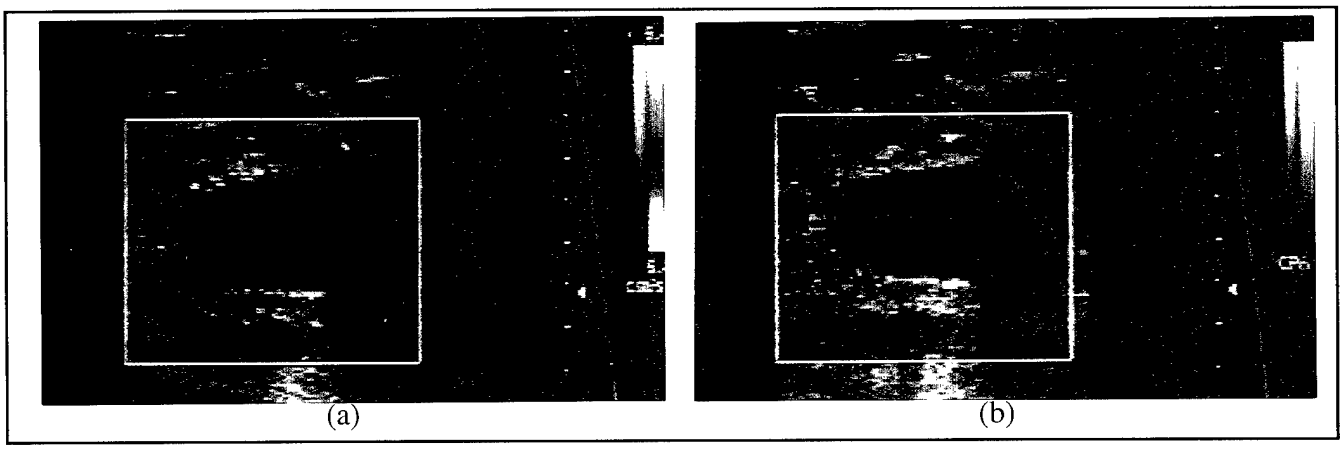


Figure 2: Doppler images of a patient with benign tumor: (a) color Doppler image, (b) power Doppler image.

The quantitative measurements of mean color level (MCL) and vascular density (VD) made on digital images are summarized in Table 1. The data is also shown as bar diagrams in Figures 3 to 5 for better appreciation of the trends.

Table 1

	MCL*				VD (% area of tumor)		
Mode -> Power Doppler	Color Doppler		Power Doppler		Color Doppler		
Region -> Periphery	Center	Periphery	Center	Periphery	Center	Periphery	Center
Malignant(M)	5.1 ± 8.5	15.7 ± 13.6	6.3 ± 10.3	8.6 ± 8.9	3.8 ± 4.8	3.1 ± 3.5	9.1 ± 10.3 9.1 ±8.4
Benign (B)	2.2 ± 4.4	6.5 ± 5.6	4.1 ± 7.4	5.1 ± 7.4	1.0 ± 1.5	1.7 ± 2.2	10.5 ± 18.4 8.8 ± 12.1
Ratio (M/B)	2.3	2.3	1.5	1.7	3.8	1.8	0.9 1.0

* Scale for MCL is 0 to 100.

The vessel count (number of vessels per mm^2) using two stains, CD31 and F8, is given in Table 2. There is a general agreement between the two stains. In most cases the F8 stain was stronger than the CD31 stain. With F8 it was easier to detect the blood vessels.

Table 2

	Histologic Vessel Count		Histologic Vessel Count	
Mode ->	CD31 stain		F8 stain	
Region -> Periphery	Center	Periphery	Center	
Malignant	58.0 ± 19.1	73.0 ± 29.9	70.6 ± 24.1	85.6 ± 33.9
Benign	43.4 ± 16.9	49.5 ± 14.5	43.7 ± 20.2	49.2 ± 19.7
Ratio (M/B)	1.3	1.5	1.7	1.7

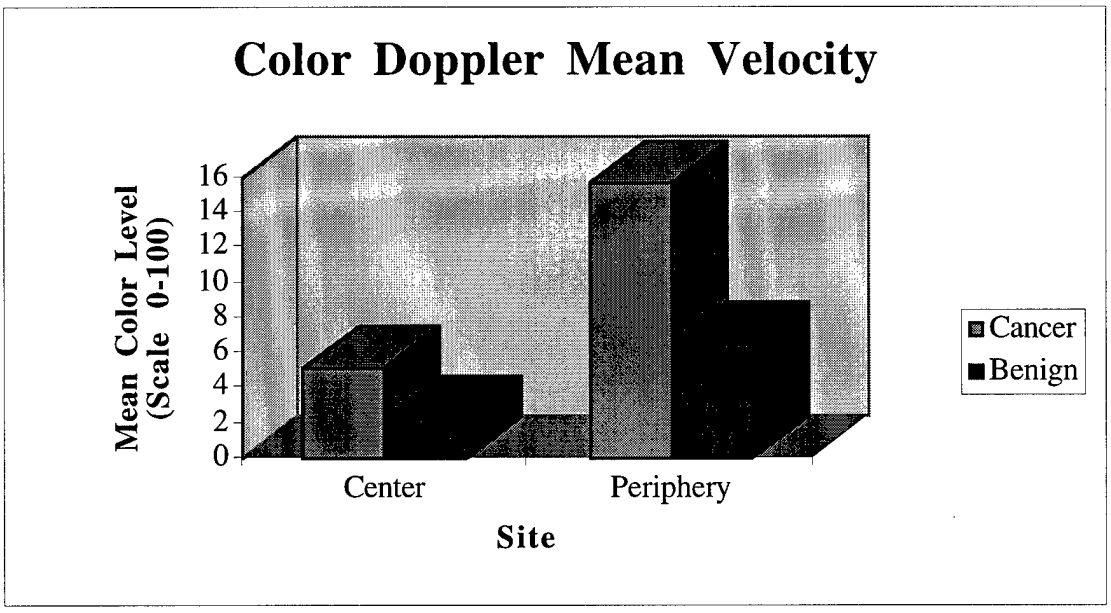


Figure 3: Comparison of MGL measured from CoD images for the malignant and benign lesions. The MGL value represents mean flow velocity on a relative scale.

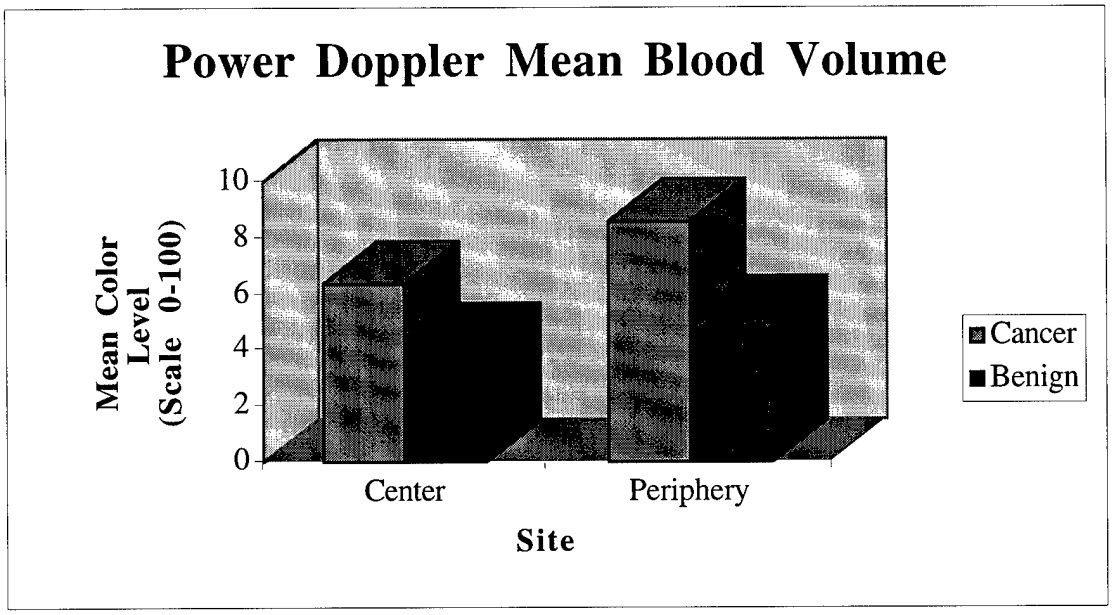


Figure 4: Comparison of MGL measured from PoD images for the malignant and benign lesions. The MGL value represents blood volume (relative scale) moving above a threshold velocity.

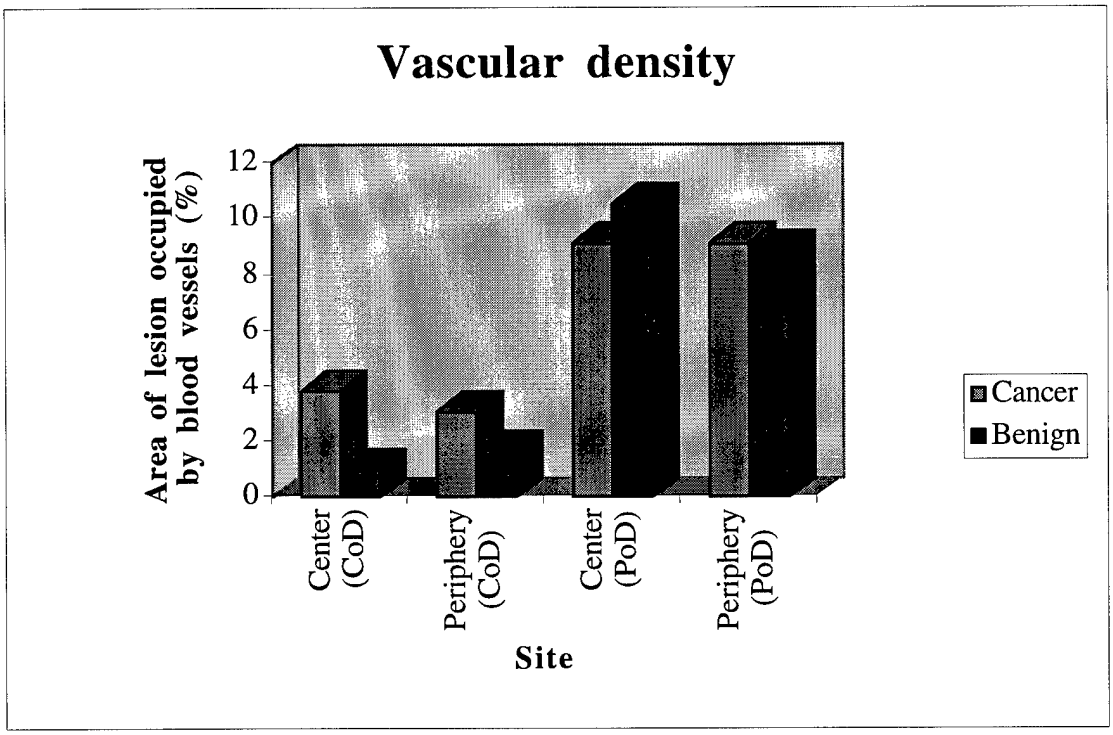


Figure 5: Comparison of vascular density measured from CoD images for the malignant and benign lesions. The MGL value represents blood volume (relative scale) moving above a threshold velocity

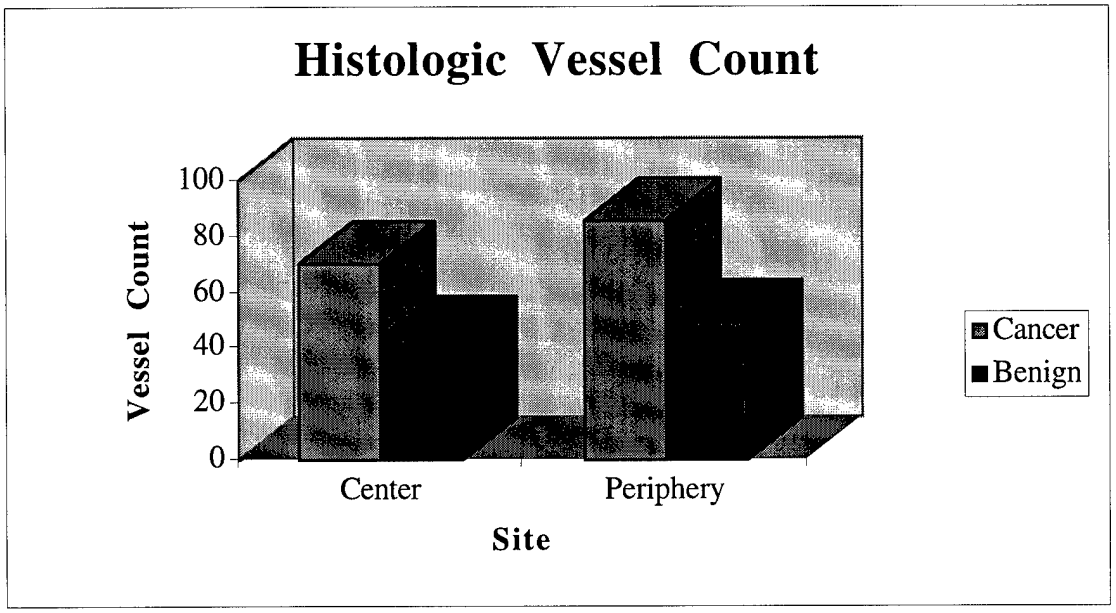


Figure 6: Histologic vessel count for the malignant and benign lesions using F8 stain.

Discussion

Flow was detected in nearly all tumor studies. These results suggest that color and power Doppler imaging have sufficient sensitivity to detect blood flow through small blood vessels of 1 mm or less in diameter. The blood flow through these vessels is slow and on the order of 1 cm/sec. Both these results, viz, small vessels and slow flow, suggest that Doppler ultrasound enables visualization of the blood vessels at the level of arterioles and venules.

The data from the color Doppler images show that the mean velocity of flow through the malignant cancer is 2.3 times greater than that in the benign lesions. This is true for both the regions, i.e., the flow through the center as well as that through the peripheral region. The velocity of flow in the peripheral region is three times larger than that in the center.

The power Doppler images represent the volume of the moving blood. These results indicate that the blood volume in a malignant is great than in a benign lesion. On average it is 1.6 times greater in the malignant lesions. In principle, the blood volume correlates with the number of blood vessels. The histologic measurements based on F8 stain show the vessel count per unit area is larger in malignant cancers as compared to benign lesions. The ratio of vessel count for malignant and benign lesion is 1.5 for the central region and 1.6 for the peripheral region. Interestingly, these ratios compare very favorably to the ratios of 1.5 and 1.7 measured for the central and peripheral regions using power Doppler imaging.

Because power Doppler measures the volume of moving blood and color Doppler measures the mean velocity, the product of the two represents flow. Figure 7 shows the calculated flow through the malignant and benign lesions. It is clear that the flow through the malignant cancers is considerably higher than that in the benign lesions. The increase in blood flow occurs both due to increase in the volume of blood and also its velocity.

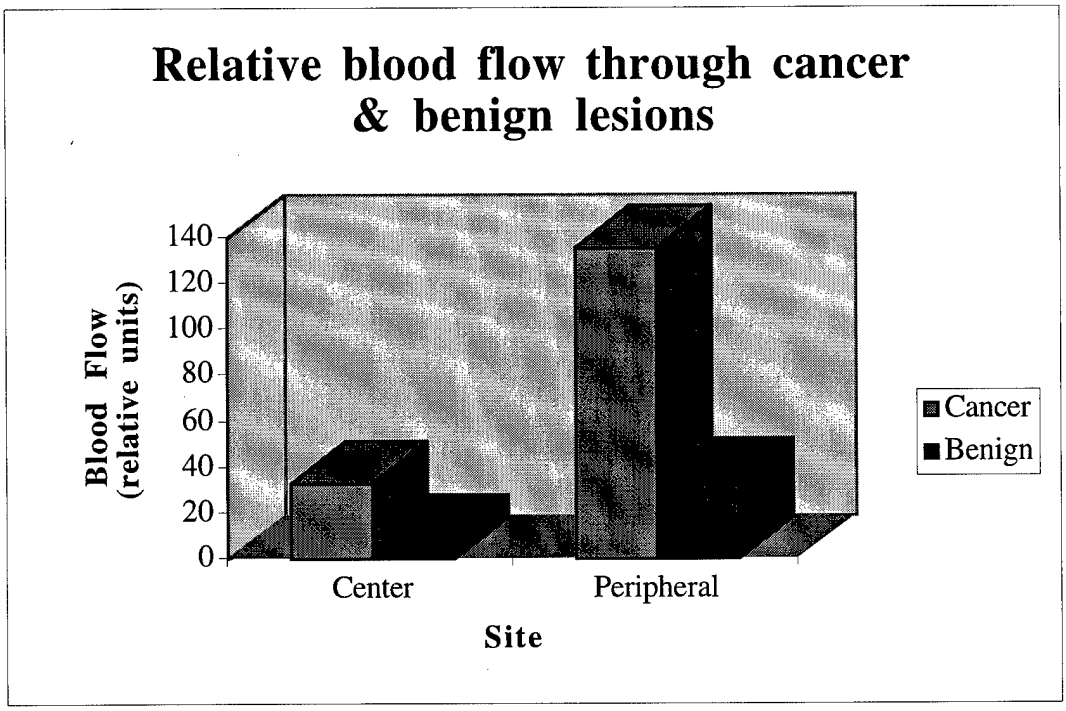


Figure 7: Comparison of blood flow for the malignant and benign lesions. These values were determined by taking the product of MCLs of CoD and POD images.

Although the results based on mean color level analysis are encouraging, the results on vascular density (VD) determined by counting pixels are mixed. The VD measurements from color Doppler images show higher density in malignant cancers than in the benign lesions. These results correlate well with the histology measurements. Quantitatively, the color Doppler measurements agree with histology for the peripheral zone but overestimates vascular density for the center. Compare the C/B ratios in Tables 1 and 2. The reason for this difference is not known. It could be due to statistical fluctuation because our data set is small, or it could be due to more profound reason related to the choice of the color-write priority. If this parameter is set too low some pixels will be artifactually counted as colored pixels. This effect would be more marked for the pixels which represent borderline signal. Tissues with higher vascularity are likely to have more of such pixels resulting in the overestimation of vascular density.

The VD measurements derived from the power Doppler images do not correlate well with the histologic measurements. We believe the primary cause for this lack of correlation is the "flash artifact". This artifact can be seen as randomly distributed color in the images show in Figure 1b. The pixels representing flash are also included by the computer analysis as representing blood vessels thus biasing the data. We intend to reanalyze this set of data by specifically choosing images that do not have "flash artifact."

It is important to note that the artifact described here consists of pixels with weak color levels. They do not significantly influence the MCL value because it is a weighted measurement and thus assigns lesser importance to pixels with low level colors.

Finally, to date our data is small and the standard deviations in the measurements are large. This means there is a considerable overlap in the values for malignant and benign lesions, even though the means are well separated. This could prove to be limiting in making decisions on a case by case basis. With the large data on hand we plan to conduct a

more detailed statistical analysis as outlined in the above proposal. If the large deviation persists we may have to segment into finer categories of malignant and benign cancers and determine which segments overlap.

In conclusion, these results suggest that MCL based measurements on Doppler images are robust and correlate exceptionally well with the histologic measurements. Thus, there is a strong suggestion, based on our initial results, that Doppler ultrasound imaging could potentially be used to measure vascularity in breast lesions noninvasively.

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